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Letter

A Chemical Approach to Introduce 2,6-Diaminopurine and 2-Aminoadenine Conjugates into Oligonucleotides without Need for Protecting Groups

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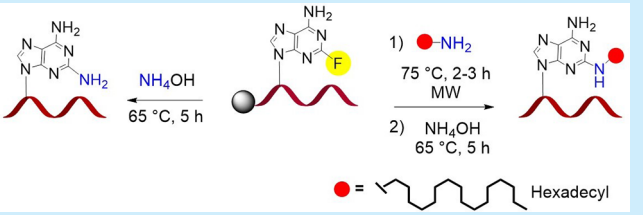
ABSTRACT: We report a simple, postsynthetic strategy for synthesis of oligonucleotides containing 2,6-diaminopurine nucleotides and 2-aminoadenine conjugates using 2-fluoro-6-amino-adenosine. The strategy allows introduction of 2,6-diaminopurine and other 2-amino group-containing ligands. The strongly electronegative 2-fluoro deactivates 6-NH_2 obviating the need for any protecting group on adenine, and simple aromatic nucleophilic substitution of fluorine makes reaction with aqueous NH_3 or R-NH_2 feasible at the 2-position.



The 2,6-diaminopurine (DAP) nucleobase was discovered in S-L2 cyanophage DNA in 1977.[1] DAP can form three hydrogen bonds with thymidine in DNA (Figure 1) and

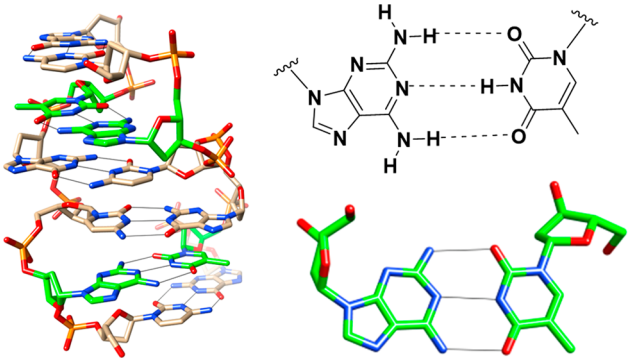


Figure 1. Three hydrogen bonds between DAP (D) and thymine as seen in the crystal structure of $[d(CDCGTG)]_2$ (PDB ID 1VTY).[2]

uridine in RNA,[2−4] enhancing duplex stability by approximately 1−2 °C per modification relative to adenine.[5,6] NMR and circular dichroism studies showed that a duplex between DNA modified with DAP and complementary RNA adopts the A-form conformation[7,8] and that the duplex formed between DNA modified with DAP and complementary DNA adopts the B-form, which transitions to the A-form in high salt.[9,10] Thus, the DAP modification does not significantly alter the conformation of nucleic acid duplexes.

The DAP modification has been studied in the context of numerous nucleic acid sugar modifications, including 2′-*O*-methyl (2′-OMe),[11−13] 2′-*O*-allyl,[14] and 2′-*O*-propargyl,[15] as well as in combination with anhydrohexitol,[16,17] threose nucleic acid,[18] locked nucleic acid (LNA),[13,19,20] unlocked nucleic acid,[21] double headed dimers,[22] 2′-5′ linked dimers,[23−25] N3′-P5′ linked dimers,[26,27] peptide nucleic acid,[28] and serinol nucleic acid.[29,30] Generally, DAP building blocks have been obtained from the guanosine analogue, but yields are low.[3,4,31−34] Synthetic routes have been developed using halogenated purines such as the 6-chloro-2-amino-purine,[11,13,14,16,17,20,35] 2,6-dicholoropurine,[36−38] and the commercially available 2,6-diaminopurine.[29,39] However, these strategies involve multiple, tedious synthetic steps. Because of the different reactivities of the two amines, the protection and deprotection strategies are difficult, and purification can be a challenge.

Starting from the free 2,6-diaminopurine containing nucleosides, various protected DAP phosphoramidites have been obtained using homoprotecting group approaches with benzoyl,[5,19] acetyl,[13] isobutyl,[13] dimethylformamidine,[40] phenoxyacetyl,[11] or Fmoc.[20,41,40] Again, because of the different reactivities of the two amines, protection and deprotection are difficult. Thus, the yields of protected DAP phosphoramidites are generally low, and deprotection is inefficient.[42] The heteroprotecting group strategy has also been employed, but synthesis becomes more complicated.[15,39]

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We previously reported the use of 2-fluoro-6-amino purines as novel precursors for DAP,[43,44] but these patent protocols have not been embraced. This prompted us to revisit this area, and herein we describe a synthetic route for simple and efficient incorporation of DAP into oligonucleotides using a postsynthetic strategy. To avoid oligonucleotide deprotection issues, we used the 2-fluoro-6-amino-adenosine monomer as the oligonucleotide building block. The combination of the fluorine inductive effect (−I) and the resonance effect (+R) of the N^6 amino meant that no protecting group was required during phosphoramidite or solid-support synthesis. We describe synthesis of 2′-deoxy, 2′-OMe, 2′-fluoro (2′-F), ribo, and LNA phosphoramidites and solid supports containing 2-fluoro-6-aminopurine (Figure 2). A postsynthetic treatment with ammonia yielded the DAP-modified oligonucleotide or, if an amine functionality was used, a 2-position conjugate.

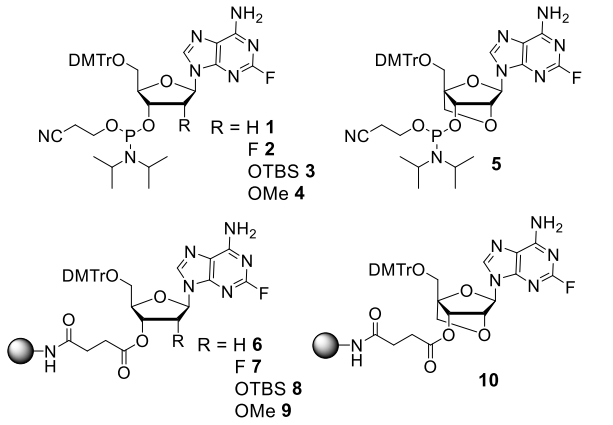
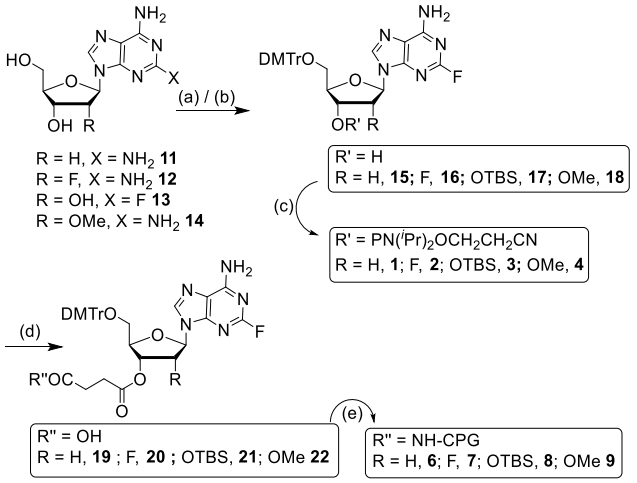


Figure 2. 2-fluoro-6-aminopurine phosphoramidites and solid supports synthesized.

Two different synthetic approaches were undertaken to afford five different 2-fluoro-6-aminopurine nucleosides depending on the available starting materials. For 2′-deoxy, 2′-F, and 2′-OMe analogues, 2,6-diaminopurine nucleosides were used as the starting material. Following the literature procedure,[45] 2-fluoro-6-aminopurine nucleosides were obtained from 2,6-diaminopurine (Schemes 1 and 2). Diazotization reactions of the diaminopurines **11**, **12**, and **14**[11] in the presence of 70% HF-pyridine and *tert*-butyl nitrite afforded the 2-fluoro analogues. Diazotization is highly selective for the 2-NH_2 vs 6-NH_2 as demonstrated earlier.[45,46]

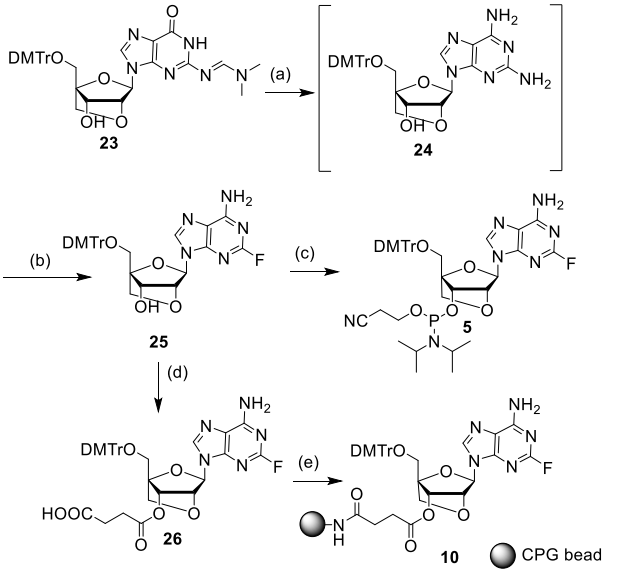
The 5′-OH groups were then protected with 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl) to afford the 5′-*O*-DMTr-2-fluoro-6-aminopurines **15**, **16**, and **18**, respectively. Subsequent phosphitylation reactions of **15**, **16**, and **18** with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite under basic conditions afforded the 2′-deoxy, 2′-F, and 2′-OMe analogues of 2-fluoro-6-aminopurine phosphoramidites **1**, **2**, and **4**, respectively, in good yields. The 2′-ribo analogue **13** was synthesized from the 2,6-diaminopurine ribonucleoside following the literature procedure.[47] The 5′-OH group of **13** was protected first with DMTr, and then the 2′-OH group was protected with TBS to afford compound **17**. The 3′-*O*-TBS-protected minor isomer was separated from the desired product **17** by column chromatography, and **17** was converted to the corresponding phosphoramidite **3** in good yield. To obtain solid supports on controlled pore glass (CPG),[48] **15**−**18** were reacted with succinic anhydride in the presence of

Scheme 1. Synthesis of Phosphoramidites 1−4 and CPGs 6−9[a]



[a]Reaction conditions: (a) (i) 70% HF-pyridine, *tert*-butyl nitrite, (ii) DMTrCl, pyridine for **15** (27%), **16** (66%), and **18** (60%); (b) (i) DMTrCl, pyridine; (ii) $AgNO_3$, pyridine, TBDMSCl, separation of 3′-isomer for **17** (17%); (c) $PClN(^iPr)_2OCH_2CH_2CN$, DIPEA, NMI, DCM, room temperature, 1 h for **1** (71%), **2** (84%), **3** (89%), and **4** (85%); (d) succinic anhydride, DMAP, DCM, room temperature, 3 h for **19** (71%), **20** (77%), **21** (93%), and **22** (78%); (e) NH_2−CPG (171 μmol/g), HBTU, DIPEA, acetonitrile for **6** (96 μmol/g), **7** (107 μmol/g), **8** (89 μmol/g), and **9** (124 μmol/g). Yields are over two steps for **15**−**18**.

Scheme 2. Synthesis of Phosphoramidite 5 and CPG 10[a]



[a]Reaction conditions: (a) (i) NaOH, MeOH, 1 M HCl, (ii) trifluoroacetic anhydride, liq. NH_3, −60 °C; (b) 70% HF/30% pyridine, *tert*-butyl nitrite for **25** (34% over two steps); (c) $PClN(^iPr)_2OCH_2CH_2CN$, DIPEA, NMI, DCM, room temperature, 1 h for **5** (85%); (d) succinic anhydride, DMAP, DCM, room temperature, 3 h for **26** (95%); (e) NH_2−CPG (171 μmol/g), HBTU, DIPEA, acetonitrile for **10** (104 μmol/g).

N,*N*-dimethylaminopyridine (DMAP) to afford compounds **19**−**22**, respectively. The carboxylic acid groups of these succinates were coupled with the free amine groups of the CPG beads under standard coupling conditions.[49,50] Un-

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reacted sites on CPG were then capped with acetic anhydride, and loading values were calculated for the resulting nucleoside-modified CPGs **6**–**9** (Scheme 1).

For the LNA analogue, commercially available guanosine nucleoside **23** was converted to corresponding 2,6-diaminopurine compound **24** and used for the next step without further purification. Compound **24** was converted to the corresponding 2-fluoro-6-aminopurine compound **25** via diazotization, followed by fluorination, with moderate yield over the two steps. Compound **25** was then converted to the corresponding phosphoramidite **5** and CPG **10** in good yields (Scheme 2).

To evaluate the efficacy of the postsynthetic conversion of 2-fluoro-6-aminopurine to DAP, we synthesized several oligonucleotides containing single or multiple incorporations using standard solid-phase synthesis methods. As models, we used previously described small interfering RNA (siRNA) targeting mouse *Ttr*,[51] single stranded antisense oligonucleotides called REVERSIRs,[52] and an antagomir targeting miR-122.[53] For oligonucleotide sequences, see Supporting Information (SI) Tables S1–S4. The standard ammonia deprotection step at 60 °C for 5 h caused displacement of 2-fluoro by ammonia and deprotection of the oligonucleotides and simultaneous cleavage from solid support, yielding the DAP-modified strands for all oligonucleotides with the exception of the oligonucleotides modified with the ribo monomer. For the oligonucleotides modified with the ribo 2-fluoro-6-aminopurine, ammonia treatment at room temperature followed by $Et_3N.3HF$ treatment resulted in 2′-silyl deprotection without 2-fluoro substitution.

To overcome this problem, a second ammonia treatment for 5 h at 60 °C was attempted; however, the second ammonia treatment led to degradation of RNA due to base hydrolysis. Therefore, to obtain the pure ribo-DAP-modified oligonucleotides, oligonucleotides were first treated with ammonia at 65 °C for 5 h followed by triethylamine trihydrofluoride treatment to yield the desired oligonucleotides (see SI Table S3).

After purification, the strands were analyzed and characterized by ion exchange chromatography and LCMS respectively. The LCMS analyses of representative strands modified with deoxy-2,6-diaminopurine (**ON5**), 2′-F-2,6-diaminopurine (**ON10**), LNA-2,6-diaminopurine (**ON15**), 2′-OMe-2,6-diaminopurine (**ON21**), and mixed 2′-F- and 2′-OMe-2,6-diaminopurine (**ON22**) demonstrated that 2,6-diaminopurine was present and that there were no unexpected modifications to other positions (Figure 3).

Delivery of RNA molecules to the appropriate tissue or cell has been a challenge. However, liver hepatocyte-specific delivery of three clinically approved siRNAs, givosiran, lumasiran, and inclisiran is made possible by conjugation to *N*-acetylgalactosamine (GalNAc), the ligand for the asialoglycoprotein receptor.[51] In earlier examples, conjugation to lipophilic molecules like cholesterol and fatty acids resulted in broad tissue distribution and cellular uptake in liver and central nervous system.[54−57] We have recently demonstrated that siRNAs can be targeted to extrahepatic tissues using a 2′-*O*-lipophile-functionalized nucleoside conjugate.[58] As fluorine is a good leaving group for the nucleophilic aromatic substitution reaction, we reasoned that 2-fluoro-6-aminopurine could serve as a site for postsynthetic conjugation of lipid amines at the 2-position of adenine of an oligonucleotide on solid support or in solution. To test this, the 2-fluoro-6-aminopurine monomer was incorporated at the 5′ end or the

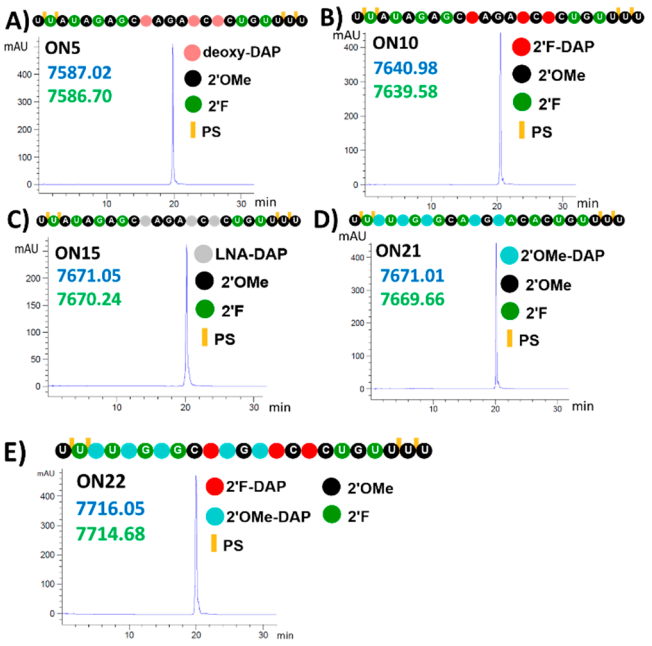

Figure 3. LCMS analysis of purified oligonucleotides (A) **ON5**, (B) **ON10**, (C) **ON15**, (D) **ON21**, and (E) **ON22** demonstrating incorporation of 2,6-diaminopurines with calculated (blue) and observed (green) masses. In bead diagrams of strands, black and green indicate 2′-OMe and 2′-F sugar modifications, respectively. Pink, red, gray, and light blue beads represent deoxy-DAP, 2′-fluoro-DAP, LNA-DAP, and 2′-OMe-DAP, respectively. Phosphorothioate linkages are indicated by a yellow vertical line.

3′ end of the *Ttr* siRNA sense strand or at position 5 of the *Ttr* siRNA antisense strand (see SI Figure S1–S2 and Tables S5 and S6). Hexadecylamine was used as the ligand (Figure 4A–C). The CPGs were first heated by microwave for 2 to 3 h. Due to high loading and the narrow space (500 Å) of the CPG pore size, 3′-end conjugation needed to be performed at 90 °C. In contrast, 5′ conjugation was completed in 2 h at 75 °C. Conjugation at an internal position was also performed at 90 °C.

Inspired by synthesis of the 2-fluoro derivative at the N^2 position of deoxyguanosine,[59] we developed a solution route to the synthesis of the 5′-end conjugate **ON62** (Figure 4D). The oligonucleotide was cleaved from solid support by treatment with concentrated NH_4OH solution for 1 h at room temperature. Under these conditions, the 2-fluoro substituent remained intact. Ammonia was evaporated from the solution, and conjugation with hexadecylamine was performed. More than 80% conjugation was achieved after 4 h at 75 °C assisted by microwave (Figure 5, see SI Figure S2 and Table S7).

In summary, we have described the synthesis of 2-fluoro-6-aminopurine nucleoside phosphoramidites and CPG building blocks with five different sugar modifications (2′-H, 2′-OH, 2′-OMe, 2′-F, and LNA) and their use in oligonucleotide synthesis. No protecting groups were required on the 2-fluoro-6-amino-adenosine. Oligonucleotides containing these monomers were also conjugated to an amine-containing lipophilic ligand to yield oligonucleotide conjugates at the position 2 of the adenine by the displacement of fluorine. Conjugation was carried out both on solid support and in solution. Work to use this conjugation strategy to attach bulky ligands, such as trivalent GalNAc,[51] in the minor groove is in progress. We also anticipate that strategic placement of conjugate within both

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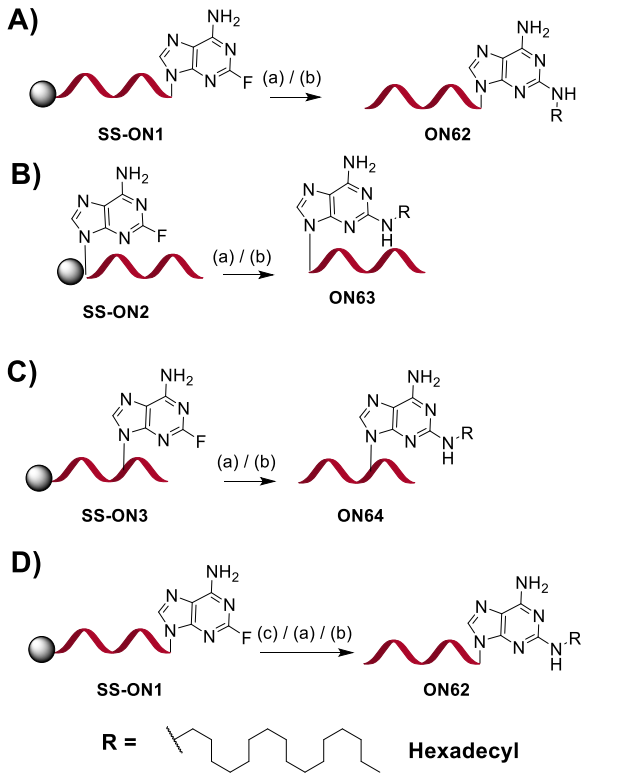

Figure 4. (A–C) Schematic of conjugation of hexadecylamine at position 2 of adenosine at (A) the 5′ end, (B) the 3′ end, and (C) an internal position of the oligonucleotide on solid support. (D) Schematic of solution-phase conjugation of hexadecylamine at position 2 of adenosine at the 5′ end of an oligonucleotide. Reaction conditions: (a) 0.1 M hexadecylamine in DMSO/EtOH/H_2O (v/v/v, 1:2:1), DIPEA, 90 °C, MW, 2–4 h; (b) 28–30% NH_4OH, 60 °C, 5 h; (c) 28–30% NH_4OH, room temperature, 1 h.

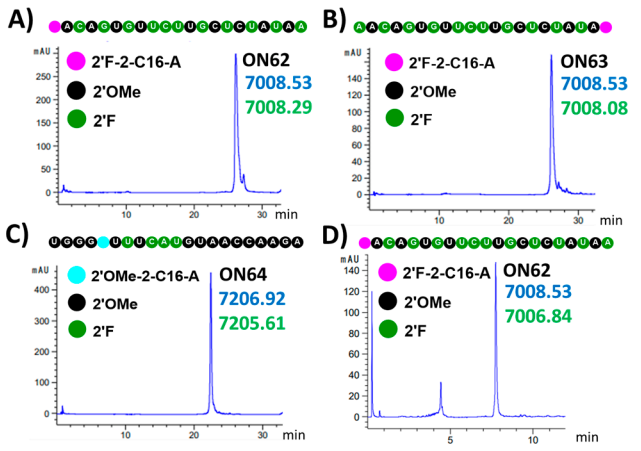

Figure 5. (A–C) LCMS analysis of purified (A) 5′-end conjugate **ON62**, (B) 3′-end conjugate **ON63**, and (C) internal conjugate **ON64** obtained after on solid support conjugation. (D) LCMS analysis of crude reaction of 5′-end conjugate **ON62** obtained by solution-phase conjugation. Calculated masses are in blue, and observed masses are in green. In the bead diagrams, black, green, light blue, and pink beads indicate 2′-OMe, 2′-F, 2-(-$NHC_{16}H_{33}$)-2′-*O*-methyl-adenosine, and 2-(-$NHC_{16}H_{33}$)-2′-fluoro-adenosine, respectively.

sense strand and antisense strands of siRNAs apart from terminal ends will be valuable.[60,61] The effect of the DAP modification on RNAi activity is also under investigation. Based on our previous work, chemical modifications are tolerated in most of the positions by the enzymes of the RNAi machinery,[61] and the stabilizing effect of DAP on duplex formation may enhance siRNA activity in strategic positions. Overall, our methodologies will enable synthesis of oligonucleotides containing DAP, various N2-position minor groove conjugates, and other modifications for therapeutic and diagnostic applications and also for structural studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.2c01848.

Experimental, and compound characterization (PDF)

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Notes

The authors declare the following competing financial interest(s): All authors, except Professor Egli and Dr. Ross, are employees of Alnylam Pharmaceuticals.

■ DEDICATION

We dedicate this work to Dr. P. Dan Cook for his contributions to nucleoside, nucleotide, and oligonucleotide based therapeutics.

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